

Molecular and parasitological study of cutaneous leishmaniasis in Bushehr province, southwest of the Islamic Republic of Iran: a cross-sectional study during 2009–2012

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Received: 5 June 2013 / Accepted: 18 October 2013 / Published online: 12 December 2013
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Abstract Cutaneous leishmaniasis (CL) is one of the most important parasitic disease in Iran. CL is distributed among more than half of 31 provinces of Iran. Studies on epidemiological aspects of the disease and *Leishmania* species identification among infected humans are necessary for providing a comprehensive prevention and control program thus; this descriptive cross-sectional study was conducted on all CL suspected patients who referred to Health Centers of Bushehr province from 2009 to 2012. Physical examinations were carried out in suspected individuals and CL cases were confirmed by microscopical examinations. Prepared slides from suspicious cases of CL were fixed with absolute methanol and stained by Giemsa 10 %. All the Giemsa-stained slides examined under a light microscope with high magnification (1,000×) and classified them based on grading of

Leishmania parasites. DNA from each slide was extracted, separately. The ribosomal internal transcribed spacer 1 was amplified with specific primers and PCR products were digested by restrict enzymes (*Hae*III), run them in 3 % gel agarose for electrophoresis and visualized on a UV transilluminator after staining with ethidium bromide. SPSS version 21 was used for data analyses. A total of 726 suspected CL cases were referred to Health Centers of Bushehr province from 2009 to 2012 and samples were only prepared from 188 of the patients whereas 43 (5.9 %) of them were microscopy positive. The most frequent of CL was observed in November (14 %) and December (12 %). The most distribution of CL lesions were observed on hands (32 %), feet (26 %), and face (21 %), respectively. The highest frequency of CL was observed in 1–9 years old (30 %). Altogether, 50 % of the patients showed one skin lesion and 2–10 skin lesions were occurred in the remained CL patients. Totally, 27 out of 43 (63 %) of the Giemsa stained slides were positive by PCR–RFLP assay because all the PCR–RFLP negative slides were prepared 3–4 years ago and kept without cover slip, and also observed scarce amastigotes during microscopy observations. *Leishmania* species were identified in 21 desirable slides which 14 of them were *L. major* and 7 of the remained isolates were identified *L. tropica* using PCR–RFLP.

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Keywords Cutaneous leishmaniasis ·
Leishmania species · PCR–RFLP · Iran

Introduction

Leishmaniasis are considered endemic in 98 countries on 5 continents: Africa, Asia, Europe, North America, and

South America (Grimaldi et al. 1987). It's estimated that 12 million are infected; furthermore above 90 % of cutaneous leishmaniasis (CL) cases are prevalent in 9 countries including Iran (WHO 2010). CL occurs in two clinical and epidemiological forms, zoonotic (ZCL) and anthroponotic (ACL). ZCL caused by *Leishmania major* is endemic in northeast, south and central areas of Iran (Nadim et al. 2008; Mohebbali et al. 2004). ACL is caused by *Leishmania tropica* and is prevalent in large and medium sized cities of Iran (Nadim et al. 2008; Hajjarian et al. 2004). Almost 20,000–30,000 cases of CL (both ACL and ZCL) are reported annually from different parts of Iran and almost 75 % of reported CL in Iran are considered being ZCL (Diseases Management Center of Iran 2009).

Multiple lesions up to more than 100 may be occurred between patients (Pearson et al. 2001). Different species of *Leishmania* are morphologically indistinguishable thus, it is often problematic using parasitological method (Marfurt et al. 2003). Identification of *Leishmania* species was performed by isoenzyme analysis, DNA sequence and monoclonal antibodies.

Using molecular and biochemical techniques, *L. infantum*, *L. major* and *L. tropica* were isolated and characterized among infected humans, animal reservoir hosts and vectors of different geographical locations of Iran (Mohebbali et al. 2002; Hajjarian et al. 2004; Alimoradi et al. 2009; Kazemi-Rad et al. 2008; Motazedian et al. 2002).

This study aimed to show the current distribution of CL in Bushehr province and identify *Leishmania* spp. which were circulating using two parasitological and molecular methods.

Materials and methods

Study area

The study was conducted in all districts of Bushehr province, southwest of Iran. This province has a strategic importance due to trading potentials, gas and oil industries and neighborhood to Arab countries of Persian Gulf through a 700 km coastal line (Fig. 1).

Study population

This study was focused on the CL records at District Health Centers of Bushehr province which the diagnosis were done based on clinical manifestations and the positive microscopical smears. The passive case detections were carried out in the Rural Health Houses (RHCs), Rural Health Centers (DHCs) and District Health Centers (DHCs) or hospitals of different districts.

The early criteria for the selection of geographical localities for microscopical examinations were the annual records of CL among indigenous population at different districts of Bushehr province during 2009–2012. Demographic characteristics and information of CL were collected by a valid questionnaire. The microscopical examinations were carried out on clinically suspicious patients who were referred to laboratories of each Health Center at different districts of Bushehr province.

Microscopical examination

Patients who had the acute lesions with at least 2 weeks duration were considered for microscopically examinations. For each case having the acute skin lesion(s). A national standard questionnaire of CL was completed and the necessary information such as name, age, gender, living place, seasonal infection, history of disease and treatment, number of lesions, lesion site and lesion appearance were collected by a valid questionnaire.

Direct smears were prepared using scraping method with a flamed sterile vaccinostyle from edge of lesions of all suspected cases. At least, two smears were prepared from the serosity material of each lesion. All the prepared slides were transferred to RHCs/DHCS laboratories.

After drying the thin smears, they fixed with absolute methanol for 1 min stained with 10 % Giemsa for 25 min. For the detection of amastigote forms of *Leishmania* sp. and confirmation of CL, microscopical examinations were performed with light microscope at 100 microscopic fields with 1,000 \times magnification. All of suspected slides were examined by two microscopic experts before recording as negative results. The confirmed cases referred to Diseases Control Units of RHCS/DHCS for receiving the appropriate treatment.

PCR–RFLP assay

DNA extraction

All the Giemsa stained slides were washed with absolute ethanol and covered with 250 μ l lysis buffer (50 mM NaCl, 50 mM Tris, 10 mM EDTA, pH 7.4, 1 % v/v Triton x-100 and 100 μ g of proteinase k per ml). DNA extraction was carried out with QIAGEN Kit according to the manufacturer's instruction. DNA samples with A260/A280 ratios were selected and stored at -20°C for further analysis.

DNA amplification by PCR

Two primers, LITSR (5-GTG CAG GAT CAT TTT CCG ATG) and L5.8s: 5-TGA TAC CAC TTA TCG CAC TT were designed for LTS1–PCR assays by identifying

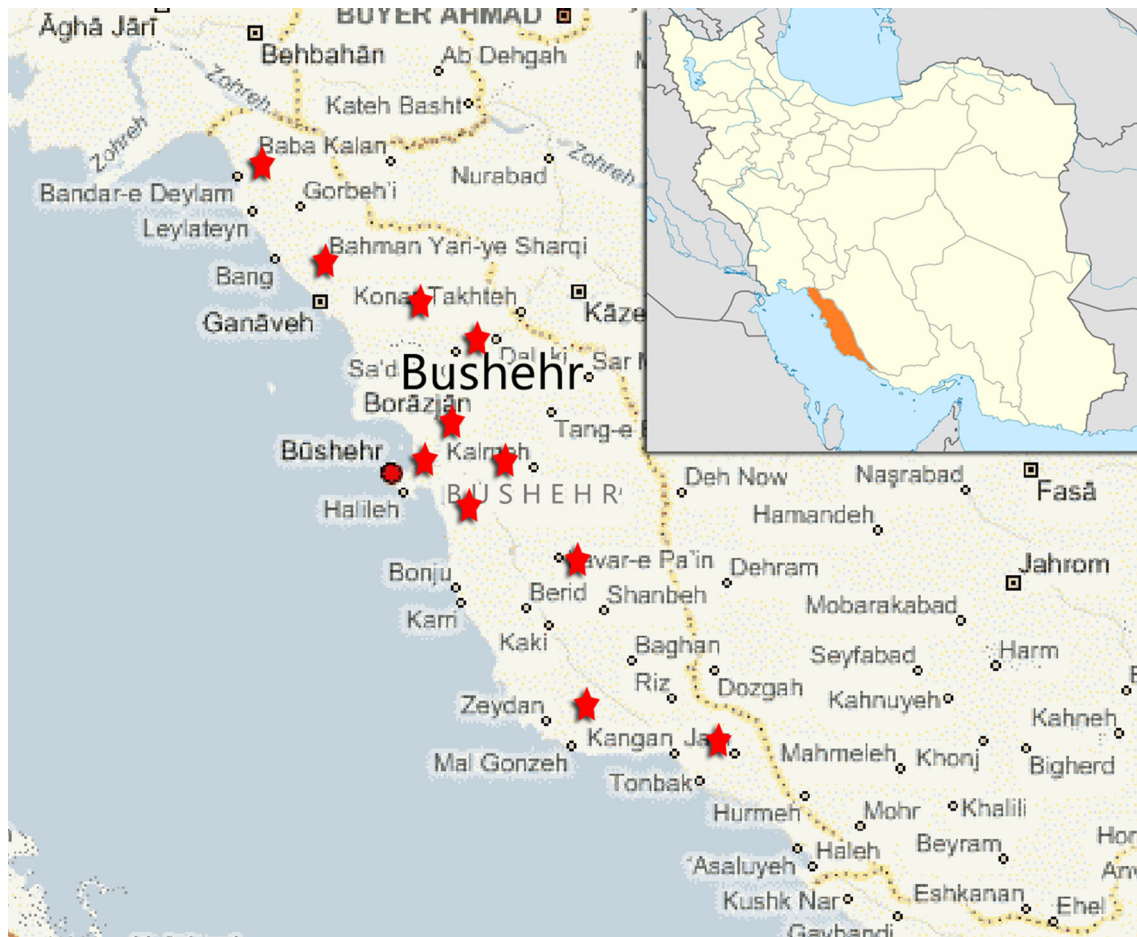


Fig. 1 Study areas from Bushehr province in southwest of Iran where this study was carried out

suitable regions described by Schonian et al. (2003) for the diagnosis and characterization of *Leishmania* in clinical samples.

PCR product analysis

Restriction fragment length polymorphism (RFLP) PCR was carried out on amplified region. Amplified products were separated by 1.5 % agarose gel electrophoresis, stained with ethidium bromide and visualized by ultraviolet (UV) transilluminator and then digital photographs were prepared. Extraction efficiencies and qualities were checked on 1 % agarose gel and DNA concentration and purity were estimated measuring the optical densities at 260 and 280 nm. We added 2 μ l *Hae*III to ITS1 PCR products (20 μ l) at 37 °C for 12 h with conditions recommended by the supplier (Fermentas, Germany). Positive specimens against kDNA were tested against the ribosomal internal transcribed spacer 1 (ITS1) region using the—GTG CAT GATCAT TTTCCG ATG) and L5.8s :5-TGA TAC CAC TTA TCG CAC TT followed by digestion by *Hae*III. A Gene Ruler TM 50 bp (base pair) DNA ladder was used

as a marker. Samples were considered as positive when a PCR product of 565 bp was detected. Positive controls containing DNA of *L. infantum*, *L. major*, *L. tropica* and a negative control containing distilled water, were included. PCR products were sequenced (MWG Company, Germany) for confirmation of PCR–RFLP results.

Data analysis

Chi squared, Mac Nemar and Fisher exact tests were used to compare prevalence values relative to gender, age, and monthly distribution. Analyses were performed with SPSS (version 21), with a probability (*p*) value of <0.05 were considered as statistically significant.

Results

Altogether, 726 suspected CL cases were referred to Health Centers of Bushehr province from 2009 to 2012 and samples were only prepared from 188 of the patients whereas 43 (5.9 %) of them were microscopy positive.

Table 1 Comparison of microscopy and PCR–RFLP results on Giemsa stained slides based on skin lesion appearance in CL patients referred to Health Centers of Bushehr province from 2009 to 2012

Clinical appearance of CL	Experiment results (positive)			
	PCR–RFLP		Microscopic	
	Percentage	Number	Percentage	Number
Wet	52	14	54	23
Dry	48	13	46	20
Total	100	27	100	43

Table 2 Comparison of microscopy and PCR–RFLP results with age groups in CL patients referred to Health Centers of Bushehr province from 2009 to 2012

Age groups	Experiment results (positive)			
	PCR–RFLP		Microscopic	
	Percentage	Number	Percentage	Number
≤9	15	4	19	8
10–19	26	7	23	10
20–29	15	4	16	7
30–39	18	5	21	9
≥40	26	7	21	9
Total	100	27	100	43

Totally, 27 out of 43 (63 %) of the Giemsa stained slides were positive by PCR–RFLP assay because all the PCR–RFLP negative slides were prepared 3–4 years ago and kept without cover slip, and also observed scarce amastigotes during microscopy observations.

Leishmania species were identified in 20 desirable slides which 14 of them were *L. major* and 7 of the remained isolates were identified *L. tropica* using PCR–RFLP (Table 1).

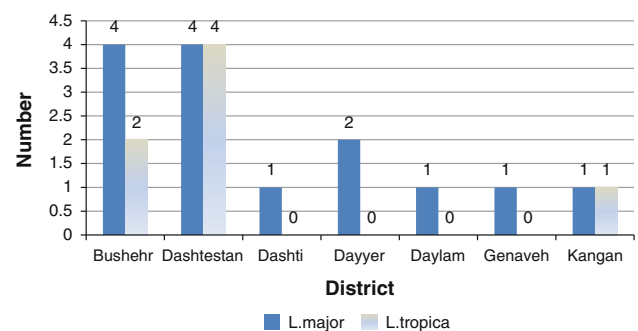
Altogether, 62.5 % of CL cases were male and 37.5 % of them were female. Age distribution of the confirmed CL patients showed in Table 2. Distribution of scar rates were 25.6 % on hands, 16.3 % on legs, 27.9 % on face, 11.6 % on hands and legs and 18.6 % on hands and face.

Totally, 46.5 % cases lived in urban areas while, 53.5 % of the patients lived in rural areas.

The number of scars on each patient varied from one to ten. Distribution of number of skin lesions were 44.2 % had one, 20.9 % had 2, 13.9 % had 3, 9.3 % had 4, and 11.6 % of remained patients had more than 4 skin lesions on their bodies.

Statistical analysis on the disease cases using SPSS program and conducting Chi square test, showed that there's not a meaningful difference between the scar numbers and the age of the patients (p value >0.05). Chi square test showed a meaningful difference between the age and permanent address and the scarred part of the cases (p value <0.05).

Chi square test didn't show statistically differences between the gender and the number of lesions (p value >0.05). This test also showed a statistically significance

**Fig. 2** Distribution *Leishmania* species in Bushehr province using the PCR–RFLP method

between the gender and living place of the patients (p value <0.05) (Fig. 2).

Results of PCR–RFLP showed that 27 out of 43 collected samples were positive by PCR–RFLP (63 %), and 43 of microscopical positive samples were showed a parasitemia from 2 to 4 grades. Twenty-one out of 27 (77.8 %) of the samples were *Leishmania* species identified that 14 of them were *L. major* and 7 of the remained isolates were *L. tropica* (Fig. 2).

Discussion

Bushehr province is one of the endemic regions for CL and VL (Hamzavi et al. 2000; Mohebbali et al. 2001). An appropriate control method for CL depends on the disease prevalence and environmental changes (WHO 2013).

Characterization of *Leishmania* species is important, because different species may require distinct treatment regimens. Furthermore, such information is also valuable in epidemiologic studies where the distribution of *Leishmania* species in human and animal hosts, as well as in insect vectors, is a prerequisite for designing appropriate control measures (Schonian et al. 2003; El Tai et al. 2000). Various biochemical, immunological, and molecular methods are invented for differential diagnosis of *Leishmania* species. Among biochemical methods, the method of analyzing iso enzymes is the most appropriate method for specifying the parasite taxonomy, but this method is time consuming and requires estimating 10–20 enzymes and also requires aseptic isolating the parasite in the specific media (Grimaldi et al. 1987). Since molecular methods such as PCR assays have been reported as desirable methods for *Leishmania* species identification (Gadisa et al. 2007).

In endemic areas where more than one *Leishmania* species is present, diagnostic tools are required for the detection of parasites directly in samples and distinguish all relevant *Leishmania* species (Schonian et al. 2003; Al-Jawabreh et al. 2006).

A Study was carried out between standardized graded direct microscopy and ITS1–PCR–RFLP on Giemsa stained smears (Al-Jawabreh et al. 2004). Also, PCR–RFLP method was used for diagnosing and typing cutaneous and visceral leishmaniasis and finally PCR–RFLP was reported as a sensitive and suitable method for routine diagnosis of leishmaniasis (Serin et al. 2007).

We used PCR–RFLP methods for diagnosis and characterization of *Leishmania* species on Giemsa stained slides without the need for cultivation them. Giemsa-stained slides are appropriate for field condition as such samples can be easily stored and sent to the diagnostic laboratory (Kazemi-Rad et al. 2008). This study showed that *Leishmania* DNA could be efficiently extracted and amplified even from old Giemsa-stained microscopic slides that were stored for 4 years with relatively high *Leishmania* spp. load and if these were protected by a cover slip. In this study, the Giemsa-stained slides were examined by both microscopy and ITS1–PCR–RFLP. Most of the slides that were high scored amastigote numbers as microscopy-positive were also positive by PCR–RFLP. This study also showed that Giemsa-stained slides used for the diagnosis with microscopy, could readily be used as samples for identifying of *Leishmania* species with PCR–RFLP. Although the costs for PCR–RFLP diagnosis are higher and its concordance is lower than microscopic examination but this method can identify *Leishmania* species without need for cultivation them. In conclusion, the PCR–RFLP method seems to be applied as a suitable tool for direct diagnosis and characterization of *Leishmania* species in Giemsa-stained slides.

Overall there were 726 CL patients who referred to the Health Centers of Bushehr province from 2009 to 2012.

Most refers were recorded during the November (14 %) and December (12 %). Seasonal changes in CL incidence are consistent with the pattern of rural type CL (Nadim 2000). Unlike the urban type which most skin lesions are seen on the face, in this study, most lesions have been observed on hands and legs and most CL cases were ≤ 9 (30 %).

In a study was surveyed CL epidemiologic situation in plains and cities of Bushehr province and detected three isolates—which were taken from patients using RAPD–PCR method—as *L. major* (Hamzavi et al. 2000).

In conclusion, differential diagnosis of CL referred to Health centers of the disease is highly recommended. Since PCR–RFLP is an accurate, sensitive, and fast method for *Leishmania* spp. characterization even on (Giemsa stained slides). Thus, we recommended this method in endemic areas of CL.

Acknowledgments This study was financially supported by Vice-Chancellors for Education and Research of School of Public Health, Med Sciences/University of Tehran as well as Bushehr University of Medical Sciences. We wish to thank Mr. Z. Zarei from Meshkin-Shahr Research station, Tehran of Medical Sciences. The authors are thankful to our other colleagues for laboratory and field activities.

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